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Comparison of microalgae and rapeseed meal as supplementary protein in the grass silage based nutrition of dairy cows

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Highlights:

- Milk production responses to microalgae were evaluated in relation to unsupplemented and rapeseed meal supplemented diets.
- Microalgae did not affect DMI or milk yield but its poorer palatability decreased the proportion of concentrate in the diet compared to rapeseed meal.
- Substitution of rapeseed meal by microalgae tended to decrease milk protein yield.
- Compared to rapeseed meal, microalgae resulted in poorer N utilisation.
- Microalgae is suitable protein feed for dairy cows, though the protein value is likely lower than that of rapeseed meal.

Abstract

Two experiments were conducted to evaluate microalgae as a protein supplement in the nutrition of lactating dairy cows in relation to unsupplemented and rapeseed meal supplemented diets. In both experiments multiparous Finnish Ayrshire cows were fed separately fixed amount of cereal-sugar beet pulp based concentrate (11 kg/d in Exp. 1 and 12 kg/d in Exp. 2), and grass silage ad libitum. In Exp. 1, six cows (212 days in milk; DIM) were used in a replicated 3×3 Latin square. Diets were supplemented isonitrogenously with rapeseed meal (pelleted rapeseed supplement, RSS), mixture of *Spirulina platensis* and *Chlorella vulgaris* microalgae (1:1 on dry matter (DM) basis; ALG) or a mixture of RSS and ALG (1:1 on crude protein (CP) basis; RSS-ALG). In Exp. 2, four intact cows and four rumen cannulated cows (190 DIM) were used in a replicated 4×4 Latin square. Treatments consisted of basal diet without protein supplement (NEG) or supplemented similarly as in Exp. 1 with the exception of RSS-ALG and ALG containing only *S. platensis.* Protein supplementation increased fibre and N digestibility but did not affect dry matter intake (DMI) or milk yield. The substitution of rapeseed by microalgae did not affect total DMI or milk yield in neither of the experiments, but changed the quality of DMI in Exp.2 by linearly decreasing concentrate:forage ratio of the diet due to poorer palatability of microalgae. The efficiency of N utilisation (NUE) in milk production varied from moderate (Exp. 1) to high (Exp. 2), and in Exp. 2 was decreased by both protein supplementation and microalgae inclusion in the diet. Protein supplementation or microalgae inclusion in the diet did not affect ruminal pH or major volatile fatty acids in Exp. 2, but both increased ruminal NH_3-N concentration. There was likely a shortage of N for rumen microbes on NEG in Exp. 2 as indicated by low milk urea N and increased microbial N flow on protein supplemented diets. In both experiments, only minor differences were observed in plasma metabolites when microalgae substituted rapeseed. Even though arterial histidine concentrations were high, arterial histidine and carnosine concentrations (Exp. 1 and 2) and milk protein yields (Exp. 2) decreased by microalgae inclusion suggesting that histidine supply may become suboptimal on microalgae supplemented diets.

Experiments demonstrated the suitability of microalgae as protein supplement for dairy cows, however, the protein value of microalgae is likely slightly lower than that of rapeseed meal.

Keywords: microalgae, *Spirulina platensis*, *Chlorella vulgaris*, rapeseed meal, dairy cow, nitrogen metabolism

Abbreviations: ALG, experimental treatment containing either the mixture of *Spirulina platensis* and *Chlorella vulgaris* (experiment 1) or *Spirulina platensis* (experiment 2); BCVFA, branched-chain VFA; NEG, experimental treatment without protein supplementation; NUE, the efficiency of N utilisation for milk protein production; RSS, experimental treatment containing rapeseed supplement; RSS-ALG, experimental treatment containing mixture of ALG and RSS.

1. Introduction

Global agricultural production is facing a tremendous challenge to match the supply of food to the rapidly increasing demand from larger and wealthier population while cutting down the environmental costs of food production and preventing undernourishment of the poorest. Schader et al. (2015) demonstrated that these goals can be achieved by reducing the use of food-competing feed components in livestock rations by using grasslands, food waste and by-products from food production as feed resources. Microalgae, which are mostly photosynthetic, unicellular or simple multicellular microorganisms growing in a widely varying environmental conditions (Mata et al., 2010), have a great potential to further reduce the food-feed competition for land. Microalgae grow extremely rapidly commonly doubling their biomass within 24 h or less (Chisti, 2007) and have a very short harvesting cycle of 1-10 days (Schenk et al., 2008). Microalgae may contain protein up to 710 g/kg dry matter (DM) (Becker, 2013) and have resulted in 5.3-20 times higher protein yields than rapeseed on area basis in Northwest Europe (van Krimpen et al., 2013). Moreover, microalgae

cultivation can be carried out in marginal or non-arable land allowing vast areas of agricultural land to be repurposed for human food (Schenk et al., 2008) or bioenergy production with significant potential to mitigate greenhouse gas emissions (Walsh et al., 2015).

Previous microalgae research on ruminants has mainly been focused on the alteration of milk fatty acid profile using algae supplements high in lipids (e.g. Boeckaert et al., 2008). In contrast, little information is available of the protein value of microalgae compared to conventional protein feeds on ruminant rations. The amino acid (AA) profile of *Chlorella vulgaris* and *Spirulina platensis*, two of the most studied, widely used and commercially available microalgae species, compares favourably to that of soybean (Becker, 2013) and rapeseed meal (Luke, 2017). However, the lower histidine content of microalgae protein than that of rapeseed protein is noteworthy, as histidine is typically the first AA limiting milk production of dairy cows on cereal and grass silage based diets (Kim et al., 1999, Vanhatalo et al., 1999). The in vitro protein degradability of *S. platensis* has been reported to be higher than that of rapeseed meal (Costa et al. 2016) which together with insufficient histidine supply might affect animal performance.

Large quantities of algae in the feed ration might also lower the palatability of the diet decreasing DM intake (DMI) (Hintz et al., 1966, Van Emon et al., 2015) and subsequently milk production can be decreased (Hristov et al., 2004). Nevertheless, compared to cottonseed meal, microalgae have resulted in similar DMI, and at high nitrogen (N) intakes, similar average daily gains of steers on low-quality forage diet (Costa et al. 2016). Protein supplementation typically increases the silage intake and milk production of dairy cows (Allen 2000, Huhtanen et al., 2011), but decreases the utilisation of feed N to milk protein, and increases the secretion of N in faeces and urine (Huhtanen et al., 2008).

The aims of these two experiments were to evaluate the effects of microalgae feeding on the dairy cow performance and N utilisation compared to diet without supplementary protein feed and diet supplemented with rapeseed meal. We hypothesised that (1) supplementary protein feed increases

DMI and milk yield but decreases N use efficiency; and (2) substitution of rapeseed meal by microalgae decreases intake of DM and histidine, milk yield and N use efficiency.

2. Materials and methods

2.1. Animals, experimental design and diets

Two studies were conducted at the University of Helsinki research farm in Helsinki, Finland. All experimental procedures were approved by the National Animal Experiment Board in Finland according to the guidelines imposed by the European Union Directive 2010/63/EU and the current Finnish legislation on animal experimentation (Act on the Protection of Animals Used for Scientific or Educational Purposes 497/2013). The cows used in the experiments were housed in individual tie stalls equipped with Roughage Intake Control system (Insentec BV, Marknesse, the Netherlands) and separate concentrate troughs, and milked twice daily at 0600 and 1700 h. In both experiments, grass silage was used as basal forage in the diet. It was preserved from the primary growth (experiment 1) or from the secondary growth (experiment 2) of timothy (*Phleum pratense*) and meadow fescue (*Festuca pratensis*) mixture. Pre-wilted grass silage was ensiled with sodium nitrite and hexamine based additive (experiment 1) applied at a recommended rate of 3 L/1000 kg (AgroSil Liquid, WK Agro Ltd., Jorvas, Finland), and with formic acid based additive (experiment 2) applied at a rate of 6 L/1000 kg (AIV 2 Plus, Kemira Ltd., Helsinki, Finland). The detailed chemical composition of the experimental feeds is given in the Table 1. Silage was offered to the animals three times (at 0900, 1400 and 1800 h) and concentrate four times (at 0600, 1100, 1700 and 1930 h) daily. The amount of concentrate given was fixed to 11 kg/d (experiment 1) and 12 kg/d (experiment 2) on fresh matter basis and grass silage was offered ad libitum to the animals to achieve 5-10 % refusals. Cows had continuous access to water. Water was added to algae (around 130 mL/kg of concentrates) before mixing it daily with other concentrate components to bind algae powder on pellets. No water was

127 added to concentrates on diets containing no algae. In addition to other concentrate components, cows
128 were offered mineral-vitamin supplement (Pihatto-Melli Plus, Raisioagro Ltd., Raisio, Finland).
129
130 *2.1.1. Experiment 1*
131 Six multiparous Finnish Ayrshire cows averaging 212 ± 30.7 d (mean ± SD) in milk were used in
132 a replicated 3 × 3 Latin square study with three different protein feed rations and three 21 d periods,
133 of which the latter 7 d formed a sampling period. At the beginning of the experiment, the cows had
134 an average milk yield of 24.8 ± 2.56 kg/d. On average, the body weight of the cows was 666 ± 53.7
135 kg at the beginning of the experiment, and 704 ± 75.4 kg at the end of the experiment.
136 The cows were randomly assigned to three dietary treatments. Treatments consisted of pelleted
137 cereal-sugar beet pulp-based concentrate (A-Rehu Ltd., Seinäjoki, Finland) supplemented with three
138 different protein feed options. These were (1) pelleted rapeseed supplement (**RSS**) (A-Rehu Ltd.), (2)
139 a mixture of two microalgae species *S. platensis* and *C. vulgaris* (1:1 on DM basis) (**ALG**) (Duplaco
140 B.V., Hengelo, the Netherlands), or (3) a mixture of RSS and ALG (1:1 on crude protein (CP) basis)
141 (**RSS-ALG**). Terms of spirulina and chlorella are later used to describe *S. platensis* and *C. vulgaris*
142 used in current experiments, respectively. Rapeseed supplement contained 767 g/kg of rapeseed meal
143 (*Brassica napus* ssp. *oleifera*) and 75 g/kg of turnip rape cake (*B. rapa* ssp. *oleifera*) (see the footnotes
144 of Table 1 for details), the protein of which was isonitrogenously substituted in half (RSS-ALG) or
145 totally (ALG) by spirulina and chlorella protein. Equal quantity of concentrate among diets was
146 adjusted with cereal-sugar beet pulp. The complete concentrate profiles and nitrogen content of
147 concentrates are depicted in Table 2.
148
149 *2.1.2. Experiment 2*
150 Eight multiparous Finnish Ayrshire cows averaging 190 ± 22.6 d in milk were used in a replicated,
151 balanced 4 × 4 Latin square study with four different dietary treatments and four 21 d periods, of

152 which the latter 7 d formed a sampling period. Four cows in one Latin square were rumen cannulated
153 (100-mm i.d.; Bar Diamond Inc., Parma, USA). At the beginning of the experiment, the cows had an
154 average milk yield of 35.8 ± 3.08 kg/d, body weight of 718 ± 54.4 kg and body condition score
155 (Edmonson et al. 1989) of 2.89 ± 0.330 in a scale of 1-5. The average body weight was 746 ± 61.7
156 kg at the end of experiment. The feeding of the animals was organized similarly as in experiment 1
157 with the exception of an additional concentrate delivery at 1430 h.

158 The four experimental treatments consisted of pelleted cereal-sugar beet pulp (A-Rehu Ltd.) based
159 concentrates without protein supplementation (negative control; **NEG**) or with three different protein
160 supplements. Those were (1) pelleted rapeseed supplement (**RSS**) (A-Rehu Ltd.), (2) spirulina (**ALG**)
161 (Duplaco B. V.), or (3) the mixture of RSS and ALG (1:1 on CP basis) (**RSS-ALG**). Rapeseed
162 supplement contained 695 g/kg of rapeseed meal (*B. napus* ssp. *oleifera*) and 138 g/kg of turnip rape
163 cake (*B. rapa* ssp. *oleifera*) (see the footnotes of Table 1 for details), the protein of which was
164 isonitrogenously substituted in half (RSS-ALG) or totally (ALG) by spirulina protein. Equal quantity
165 of concentrate among diets was adjusted with cereal-sugar beet pulp. Small amounts of molasses
166 (Suomen Rehu Ltd., Hyvinkää, Finland) and molassed sugar beet pulp (Suomen Rehu Ltd.) were
167 added to ALG and RSS-ALG diets to compensate for the contribution of these ingredients in rapeseed
168 supplement. The composition and nitrogen content of concentrates is described in Table 2.
169

170 *2.2. Measurements and sampling*

171 Feed intake and milk yield of the cows were recorded daily throughout the experiment. However,
172 only measurements on d 15-21 of each period were used for statistical analysis. During the
173 measurement period, representative samples of diet ingredients were collected daily, combined by
174 period to provide a composite sample for chemical analysis and stored at -20 °C until analyses.
175 Refusal concentrates were weighed daily during the collection period and combined by cow within
176 period to provide a composite sample for DM determination and stored at -20 °C until analysis.

In experiment 2, samples of ruminal fluid (approximately 100-150 ml) from rumen cannulated cows were collected on d 20 at 0600, 0730, 0900, 1030, 1200, 1330, 1500 and 1630 h via the rumen cannula. The ruminal fluid was filtered through a single layer of cheesecloth and pH was immediately measured with electronic pH meter (S20 SevenEasyTM pH, Mettler-Toledo Ltd, Leicester, Great Britain). Three subsamples were taken from the filtered ruminal fluid. For later determination of volatile fatty acid (VFA) concentrations, a subsample of 5 ml of ruminal fluid was preserved with 0.5 ml of saturated mercury (II) chloride and 2 ml of 1 mol/L sodium hyroxide and stored at -20 °C. Subsamples of ruminal fluid (15 ml) destined for the determination of NH_3-N were preserved with 0.3 ml of 9 mol/L sulphuric acid and stored at –20 °C.

In both experiments blood samples were taken from the superficial epigastric (mammary) vein and coccygeal (tail) vessel of all cows except for intact cows from of which samples were taken only from tail vein in experiment 2. Blood samples were collected on d 21 at 0530, 0830 and 1130 h and treated similarly as in Puhakka et al. (2016). Milk samples were collected from all experimental cows over four consecutive milkings, starting on d 18 at 1700 h. Milk samples were preserved with Bronopol broad spectrum microtabs (Valio Ltd., Helsinki, Finland) and analysed for fat, CP, lactose and urea by mid-infrared spectroscopy (Milko-Scan 605, Foss Electric, Hillerød, Denmark) in commercial laboratory (Valio Ltd., Seinäjoki, Finland).

Spot samples of faeces were obtained from the rectum of each cow at 0700 and 1600 h on d 17-20 of each period, composited by cow within period and stored frozen (-20 °C) until analyses. In the experiment 2, spot samples of urine (minimum of 500 mL) were obtained by mild manual stimulation of the vulva on d 18 at 0530 and 1430 h and on d 19 at 1000 and 1900 h. Fresh samples were acidified with 15 mL of 5 mol/L sulfuric acid and treated similarly as in Puhakka et al. (2016) for analysis of purine derivatives (allantoin, creatinine and uric acid) and urea-N. Concentration of N was determined from undiluted, acidified urine. Cows were weighed on two consecutive days at the beginning and end of the experiment (CV 9600 Scale, Solotop Ltd., Helsinki, Finland).

202

203 *2.4. Chemical analysis*

204 DM and organic matter (OM) content of the feeds, feed refusals and faeces were determined as
205 reported by Salin et al. (2012). Water soluble carbohydrate and in vitro digestible OM in DM
206 (DOMD) content of silages, neutral detergent fibre (NDF) content of feeds and faeces, and
207 indigestible NDF (iNDF) content of silage were determined with the same methods as reported by
208 Puhakka et al. (2016). In NDF-analysis, crucibles with pore size of 40-100 µm were used for all
209 samples and heat stable amylase for analysis of concentrate components. Results of NDF are
210 expressed inclusive of residual ash. Kjeldahl-N content of the feeds, faeces and urine was determined
211 as reported by Puhakka et al. (2016) and CP content of the feeds was calculated as Kjeldahl-N×6.25.
212 The DM content of silages was corrected for the loss of volatile compounds (lactic acid, VFA and
213 NH_3-N) according to Huida et al. (1986), the concentrations of which were analysed as reported by
214 Puhakka et al. (2016).

215 Acid insoluble ash (AIA) was analysed by acid hydrolysis and used as an internal marker to
216 determine total tract apparent digestibility of the diets and nutrients (Van Keulen and Young, 1977).
217 For the analysis of crude fat, concentrate samples were hydrolysed with 800 mL of HCl (4 mol/L)
218 (SoxCap 2047 hydrolysis unit, FOSS Analytical, Hillerød, Denmark) following an extraction with 90
219 mL of petroleum ether (FOSS Soxtec 8000 extraction unit, FOSS Analytical, Hillerød, Denmark).

220 For the analysis of AA, feed samples were hydrolysed and analysed as reported by Puhakka et al.
221 (2016). Nomenclature of International Union of Pure and Applied Chemistry (IUPAC) has been used
222 for the naming AA. Terms Nπ (nitrogen atom closest to the side chain) and Nτ (nitrogen atom furthest
223 from the side chain) are used later on to describe the position of methylated nitrogen atoms in the
224 imidazole ring of histidine, according to the IUPAC recommendations. Thus, 3-methylhistidine, the
225 product of muscle actin and myosin catabolism will be referred to as Nτ-methylhistidine, and 1-
226 methylhistidine, the product of anserine breakdown, will be referred to as Nπ-methylhistidine.

The VFA concentrations of ruminal fluid were determined as follows. Rumen fluid sample was filtrated through 0.22 µm filter. The filtrate (300 µL) was diluted with 150 µL 2-ethylbutyricacid (internal standard in acetonitrile-1.5 mol/L H_3PO_4 (1:1) solution) and 150 µL of 0.533 mol/L HCl. Diluted sample (20 µL) was added to a vial followed by addition of 40 µL of 100 mmol/L 2-(trifluoromethyl)-phenylhydrazine in 0.1 mol/L HCl-acetonitrile (1:1) solution. The solution was shaken for 5 seconds by vortex shaker and 40 µL of 250 mmol/L activation reagent (1-ethyl-3-(3-dimethylaminopropyl)) carbodi-imide in ethanol containing 3% of pyridine was added to reaction vial. After shaking, the reaction vial was heated for 30 min at 60 °C. Liquid chromatography and data analysis was performed similarly as reported earlier for analysis of silage VFA (Puhakka et al., 2016).

Plasma concentrations of acetic acid, BHBA and AA were analysed as reported by Puhakka et al. (2016), and glucose, non-esterified fatty acids (NEFA) and insulin as reported by Salin et al. (2012). The concentrations of purine derivatives in urine samples were analysed as reported by Puhakka et al. (2016) with ultra-performance liquid chromatography (Waters Acquity UPLC; column 186003540, Acquity UPLC HSS T3, Waters Corporation). Urea-N concentration of urine was determined by colorimetric enzyme kit (UREA liquicolor, 10505, Human Gesellschaft, Wiesbaden, Germany) with UV-spectrophotometer (Shimadzu UV-VIS mini 1240, Shimadzu Europa GmbH, Duisburg, Germany) according to the manufacturer's instructions.

2.5. Calculations and statistical analysis

Daily DMI was calculated as the difference between DM offered and DM residue. Energy corrected milk (ECM) was calculated according to Sjaunja et al. (1991). The metabolisable energy (ME) content of experimental concentrates other than microalgae was based on information given by feed manufacturers (see the footnotes of Tables 4 and 6 for details). The ME content of microalgae was estimated based on the equation:

ME (MJ/kg DM) = [15.2 × digestible CP (g/kg DM) + 34.2 × digestible crude fat (g/kg DM) + 12.8 × digestible crude fibre (g/kg DM) + 15.9 × digestible nitrogen free extract (NFE; g/kg DM)] / 1000 (MAFF, 1984).

The crude fibre content of spirulina and chlorella was assumed to be zero based on zero NDF concentration in current experiments and the NFE content of microalgae was determined by difference of other macronutrients. In all microalgae species, the digestibility coefficients of CP (0.738), ether extract (0.625) and NFE (0.670) were based on Hintz et al. (1966). Resulting ME contents were 10.9 and 10.8 MJ/kg DM for spirulina in experiment 1 and 2, respectively, and 11.4 MJ/kg DM for chlorella in experiment 1. The ME content and intake of the silages was calculated according to Finnish nutrient requirements (Luke, 2017). ME requirements for maintenance (MJ/d) and milk production (MJ/d and MJ/kg of ECM) were calculated as live weight $(kg)^{0.75}$ × 0.515 + ECM yield (kg/d) × 5.15 (Luke, 2017), taking into account the effect of pregnancy on ME requirements and ignoring the changes in live weight during the experiment. ME balance of animals was calculated as a difference of ME intake and ME requirements.

Microbial protein yield in the rumen and daily urine volume was estimated indirectly based on urine purine derivatives assuming the creatinine excretion rate of 25 mg/kg of BW as reported by Puhakka et al. (2016). Mammary plasma flow was estimated according to the application of Fick principle based on the stoichiometric transfer of mammary Phe and Tyr uptake into milk (Cant et al., 1993) as reported by Vanhatalo et al. (1999).

Experimental data were subjected to analysis of variance using Mixed-procedure of SAS 9.3 version (Statistical Analysis Systems Institute Inc., Cary, NC, USA). The statistical model for both experiments was as follows:

$$Y_{ijklm} = \mu + A(S)_i + S_j + P(S)_k + D_l + E_{ijklm},$$

where Y_{ijklm} is dependent variable, μ is overall mean, A is the effect of animal, S is the effect of block, P is the effect of period, D is the effect of experimental diet and E is the random residual error. Block,

period within block and diet were considered as fixed effects and animal within block as a random effect. In experiment 2, measurements of rumen fermentation characteristics were subjected to analysis of variance for repeated measures with model as follows:

$$Y_{ijklm} = \mu + A_i + P_j + D_k + T_l + APD_{ijk} + AT_{il} + PT_{jl} + TD_{kl} + E_{ijklm,}$$

where Y_{ijklm} is dependent variable, μ is overall mean, A is the effect of animal (random effect), P is the effect of period (fixed effect), D is the effect of experimental diet (fixed effect), T is the effect of sampling time (fixed effect), APD is the interaction of A, P and D (random effect), AT is the interaction of A and T (random effect), PT is the interaction of P and T (fixed effect), TD is the interaction of T and D (fixed effect) and E is the random residual error. The degrees of freedom were calculated according to the Satterthwaite method. The covariance structure AR(1) was applied with the interaction of animal and period as the subject for repeated measures. In the presence of T×D interactions, data from individual sampling times was further statistically analysed with a simplified model with animal as a random effect, and period and diet as fixed effects. Otherwise only least squares means of treatment effects on rumen fermentation characteristics were presented. Same simplified model was used for statistical analysis also when data from only one block was involved (mammary uptake of plasma metabolites and AA in experiment 2).

P-values ≤ 0.05 were regarded as significant, and $0.05 < P \leq 0.10$ were accepted as a tendency. In both experiments, sums of squares of the treatment effects were further separated into single degree of freedom comparisons using polynomial contrasts. Linear and quadratic polynomials were constructed to test the effect of replacing rapeseed protein with microalgae protein. In addition, the significance of protein supplementation (RSS + RSS-ALG + ALG vs. NEG) was tested in the experiment 2. Logarithmic or squared transformations were used to correct for deviations from normality and homoscedasticity of residuals. If transformations were needed, least squares means are reported from statistical analysis of untransformed values and SEM and P-values from analysis of transformed data.

3. Results

3.1. Diet composition

The chemical composition of feeds in experiments 1 and 2 is depicted in Table 1. The concentration of fermentation acids and the proportion of NH_3-N in total N was low in grass silage in both experiments (see the footnotes of Table 1 for details). The CP content and in vitro DOMD of silages were relatively low in both experiments.

In contrast to other experimental feeds, no NDF was detected in spirulina and chlorella. The protein content of spirulina and chlorella was markedly higher than that of rapeseed supplement. The protein feeds also differed in AA composition (Table 3), especially in histidine, lysine, isoleucine and leucine concentrations, histidine being highest in rapeseed supplement, lysine being lowest in spirulina and the isoleucine and leucine being highest in spirulina. Generally, the essential AA (EAA) profile of chlorella was closer to that of rapeseed supplement than spirulina, excluding histidine.

3.2. Animal measurements

3.2.1. Experiment 1

Intakes of dietary components, nutrients and AA, apparent digestibility of nutrients, and milk yield and composition are presented in Table 4. Silage and diet DM intake were not affected ($P>0.05$) by substitution of rapeseed supplement by microalgae. Inclusion of microalgae in the diet slightly increased CP concentration of the experimental diets consumed ($P<0.001$) (Supplementary Table 1) and CP intake ($P=0.009$). Substitution of rapeseed supplement by microalgae linearly increased the intake of EAA ($P=0.004$), and many single AA ($P\leq0.047$), excluding histidine intake that linearly decreased ($P=0.003$) and tryptophan with no change. Similar pattern was observed on AA concentration of the diets consumed (Supplementary Table 1).

Treatments had no effect on apparent digestibility of DM, OM, NDF or CP (Table 4). Milk production was on average 23.2 kg/d and ECM production 25.6 kg/d. Milk yield tended to change in a quadratic manner being highest (P=0.084) on RSS-ALG. However, treatments had no effect on fat or ECM yield (P>0.05). Only few differences between treatments were found on arterial concentrations of plasma metabolites and AA (Table 5). Arterial BHBA concentrations exhibited a quadratic pattern (P=0.011) being lower on RSS-ALG than on RSS and ALG. Substitution of rapeseed supplement by microalgae linearly decreased arterial concentrations of histidine (P=0.012) and carnosine (P=0.022). Mammary plasma flow and uptakes of all plasma metabolites including AA are presented in Supplementary Table 2 with no significant effects concerning energy metabolites or EAA.

3.2.2. Experiment 2

Intakes of dietary components, nutrients and AA, apparent digestibility of nutrients, and milk yield and composition are presented in Table 6. The supplementary protein in the diet tended to increase (P=0.071) silage intake, increased (P=0.036) ME intake, and decreased (P=0.034) the proportion of concentrate in the diet. Addition of protein supplement also increased intake of CP (P<0.001) and CP concentration of the diet consumed (P<0.001) (Supplementary Table 3). The CP concentration of the diet consumed was also linearly increased (P<0.001) by inclusion of spirulina in the diet, however, CP intake was not affected (P>0.05). The substitution of rapeseed supplement by spirulina linearly decreased (P=0.044) the proportion of concentrate in the diet, and linearly increased (P=0.021) ME balance.

The supplementary protein in the diet increased the intake of all EAA (P<0.001). Substitution of rapeseed supplement by spirulina linearly increased or tended to increase the intake of BCAA (P=0.003), EAA (P=0.020), NEAA (P=0.051), and many single AA (P≤0.080), excluding histidine and tryptophan intakes, which linearly decreased (P≤0.004), and lysine that was unaffected (P>0.05).

Corresponding responses were observed on AA concentrations of the diet consumed (Supplementary Table 3).

Protein supplementation increased the digestibility of DM (P=0.006), OM (P=0.003), NDF (P<0.001) and CP (P<0.001) (Table 6). However, the source of protein feed did not have an effect on digestibility parameters (P>0.05). Milk yield was not affected (P>0.05) by the addition or source of protein feed. Protein supplementation increased (P<0.001) milk urea N (MUN) concentration. The substitution of rapeseed supplement by spirulina tended to linearly decrease (P=0.059) milk protein yield. The urea N content of the milk was higher (P=0.028 for quadratic effect) and the lactose content of the milk tended to be lower (P=0.095 for quadratic effect) on RSS-ALG compared to RSS and ALG. Also the efficiency of milk production in terms of ECM yield (kg/d) to DM intake (kg/d) ratio was lower for RSS-ALG than for RSS and ALG (P=0.049 for quadratic effect).

Sampling time had no significant effect on ruminal fermentation characteristics, except for NH_3-N and isovalerate, thus only least squares means of treatment effects are presented in the Table 7. Protein supplementation did not affect rumen pH, but increased rumen NH_3-N concentrations which were higher on protein supplemented than NEG diets for the majority of time between feedings ($P \leq 0.014$ for time×diet interaction; Figure 1). Both the addition and source of protein feed had only minor effects on the molar proportions of VFA in the ruminal fluid with supplementary protein increasing those of isobutyrate (P=0.026), and caproate (P=0.022) and spirulina inclusion in the diet increasing that of isobutyrate (P=0.034). However, protein supplementation increased the molar proportions of isovalerate especially during the first hours after the concentrate feeding (< 6 h), and these proportions were increased especially by spirulina inclusion in the diet ($P \leq 0.043$ for time × diet interaction; Supplementary figure 1).

Microbial N production tended to increase (P=0.066) by protein supplementation (Table 8). N balance was positive on all treatments and tended to linearly increase (P=0.075) when rapeseed supplement was substituted by spirulina. Proportion of N secreted in milk was decreased by protein

supplementation (P<0.001) and spirulina inclusion in the diet (=0.021). In addition, protein supplementation decreased (P<0.001) the proportion of N excreted in faeces and increased (P<0.001) that in urine as well as urine excretion (P=0.002) and proportion of urinary N excreted as urea (P<0.001).

Arterial concentrations of plasma metabolites and AA are presented in Table 9. The substitution of rapeseed supplement by spirulina increased linearly (P=0.033) arterial NEFA concentration and tended to linearly increase (P=0.096) that of BHBA. Arterial insulin concentrations exhibited a tendency (P=0.071) on quadratic pattern being highest on RSS-ALG. Mammary plasma flow and uptakes of plasma metabolites and AA are presented in Supplementary Table 4. Mammary uptake of glucose was increased by protein supplementation (P=0.016) and it was lower (P=0.004 for quadratic effect) on ALG than on RSS and RSS-ALG when spirulina substituted rapeseed supplement in the diet.

Protein supplementation increased arterial concentrations of BCAA (P=0.002) and EAA (P=0.002), and tended to increase that of total AA (P=0.087) (Table 9). In addition, the arterial concentrations of all single EAA (except for tryptophan) were increased (P≤0.030) or tended to increase (P=0.096; Phe) by protein supplementation. In contrast, protein supplementation decreased or tended to decrease arterial concentrations of carnosine (P=0.059), β-alanine (P=0.018), Nτ-methylhistidine (P<0.001), and Nπ-methylhistidine (P<0.001). Microalgae inclusion in the diet linearly decreased (P=0.006) arterial concentrations of carnosine and tended to decrease (P=0.081) that of histidine. Protein supplementation increased (P≤0.044) mammary uptake of Leu, Phe, Val, BCAA, and EAA (Supplementary Table 4).

4. Discussion

4.1. Microalgae composition

399 The microalgae used in current experiments had very high CP concentration at a typical level of
400 around 700 g/kg DM for spirulina (Becker, 2013, Panjaitan et al., 2015, Costa et al., 2016) and around
401 500-600 g/kg DM for chlorella (Becker, 2013). The crude fat concentration was quite low (< 96 g/kg
402 DM) especially on spirulina compared to the microalgae often used in ruminant experiments focusing
403 on alteration of milk fatty acid profile (e.g. 581 g/kg DM in DHA-enriched *Schizochytrium*
404 sp., Boeckaert et al., 2008). Spirulina and chlorella in current experiments did not contain any NDF.
405 Drewery et al. (2014) reported similar results for lipid extracted *Chlorella* sp., whereas for spirulina
406 low NDF concentrations of 35-63 g/kg DM have been reported (Panjaitan et al., 2015, Costa et al.,
407 2016).
408
409 *4.2. Feed intake, digestibility and milk production*
410 Contrary to our hypothesis, total DM intake was not affected by supplementary protein or the
411 substitution of rapeseed supplement by the mixture of spirulina and chlorella (experiment 1) or
412 spirulina (experiment 2). It should be, however, noted that in experiment 2 the complete substitution
413 of rapeseed supplement by spirulina changed the quality of DMI. Due to incomplete concentrate
414 intake, the proportion of concentrate in the diet linearly decreased by spirulina inclusion, the
415 concentrate intake being 0.94 kg/d lower on ALG compared to RSS. Consequently, cows
416 compensated for the lower palatability of concentrates containing spirulina by numerically increasing
417 silage DM intake (+0.40 kg/d on ALG compared to RSS), leading to unaffected total DMI. According
418 to Hintz et al. (1966) and Van Emon et al. (2015), large quantities of microalgae may decrease the
419 acceptability of diet on wethers and beef steers, respectively, but this has not been observed in
420 microalgae experiments with *Bos indicus* steers (Costa et al., 2016). The lower acceptability of
421 microalgae by animals might be caused by the taste and odour properties, nutritive characteristics or
422 physical structure of dry powdery microalgae. The dry appearance of microalgae was unlikely the
423 cause of poor palatability of microalgae diets in experiment 2 as a small amount of water was added

to concentrates containing microalgae to bind powdery algae on pellets, resulting in an average DM content of 755 and 783 g/kg of microalgae concentrates in experiments 1 and 2, respectively. To some extent, though, this caused the breakdown of the pelleted structure of concentrates which in turn might have affected voluntary concentrate intake. Hintz et al. (1966) noted that the impaired palatability of microalgae could be avoided by pelleting the dietary ration.

Lack of DMI response to protein supplementation in experiment 2 contradicts the common perception that protein supplementation increases DMI irrespective of protein source (Huhtanen et al., 2011). The increase in DMI is suggested to relate to faster rate of fibre digestion in the rumen (Oldham, 1984) and metabolic effects, such as improved AA to ME ratio at the tissue level (Huhtanen et al., 2011). Indeed, the apparent digestibility of nutrients was improved by protein supplementation in experiment 2. The improvements of OM and NDF digestibility by protein supplementation were on average 0.58 and 1.8 g/kg per 1 g/kg DM increase of diet CP concentration, respectively, being larger than the respective increases of 0.31 and 0.64 reported for rapeseed meal in the meta-analysis of Huhtanen et al. (2011). The pronounced digestibility responses in current experiment were likely caused by the low CP and DOMD concentration of silage, and CP concentration of NEG, which were much lower in the current experiment than on average in the diets used in the meta-analysis of Huhtanen et al. (2011).

The cell wall of spirulina, a cyanobacterium, consist mainly of murein (peptidoglycan) (Lee, 2008). The exact cell wall composition of chlorella remains unclear, but the digestibility of chlorella is suggested to be mainly determined by proteinaceous polymers rather than carbohydrates (Mahdy et al., 2015). These findings are in agreement with zero NDF concentration of microalgae observed in our experiments, although it can also be questioned whether standard NDF determination is a suitable analytical method for unicellular microalgae with very small particle size. Despite of the differences in NDF concentration between protein feeds, microalgae inclusion in the diet did not affect NDF intake, most likely because of the simultaneous changes in silage and cereal-sugar beet

449 pulp intake. Due to the differences in cell wall composition, cyanobacteria may be more easily
450 fermented and digested than chlorella as indicated by the results of anaerobic digestion of algal
451 biomass for biogas production (Mendez et al., 2015). This is also supported by the higher in vitro
452 rumen protein degradability of spirulina than that of another species of *Chlorella* family, *C.*
453 *pyrenoidosa* (Costa et al., 2016). Further studies are needed for better understanding of the exact
454 digestion process and passage kinetics of unicellular microalgae as well as the possible differences
455 between different microalgae species.

456 Milk yield was not affected by protein supplementation, which is in agreement with the notion
457 that DMI is typically the main factor affecting milk and milk protein yield (Hristov et al., 2004). Also,
458 the low milk production response to microalgae might be partly explained by the decrease of
459 concentrate proportion in the diet. However, there was a tendency for increased ECM and fat yield
460 by protein supplementation, which might reflect the increases in nutrient digestibility and supply of
461 AA. Milk (2.3 kg) and milk protein (82 g) responses per 1 kg increase in CP intake obtained on RSS
462 were lower than the corresponding values of 3.4 kg and 136 g on rapeseed meal supplemented diets
463 in the meta-analysis of Huhtanen et al. (2011), but agreed with the results of Puhakka et al. (2016).
464 The corresponding responses were even lower for diets containing microalgae, being 0.96 and 0.95
465 kg of milk, and -2.41 and 6.40 g of milk protein for RSS-ALG and ALG, respectively. The low
466 responses in the present study were probably not related to the late lactation stage because Saarisalo
467 et al. (2002) reported that protein supplementation in late lactation had an equal milk production
468 response as compared to early or mid-lactation (Huhtanen, 1998). In fact, low milk production
469 response to protein supplementation might be partly explained by the decrease of concentrate
470 proportion in the diet observed on microalgae containing diets.

471

472 *4.3. Energy and nitrogen metabolism*

Rumen fermentation pattern observed in experiment 2 with low molar proportion of propionate and high molar proportions of lipogenic VFA in rumen VFA is typical to diets based on restrictively fermented grass silage (Huhtanen, 1998). Lacking responses in major VFA to protein supplementation are in agreement with Korhonen et al. (2002) and in accordance with the constant plasma glucose concentration and milk fat content across all treatments in our experiments.

Positive calculated ME balance suggest that the energy supply of cows was adequate. However, the ME value of grass silages (10.6 MJ/kg DM) was moderate based on Finnish nutrient requirements (Luke, 2017), indicating that insufficient energy supply might have limited the growth of rumen microorganisms and the utilisation of supplementary protein (Huhtanen and Hristov, 2010) as reflected with the low milk production response to supplementary protein in experiment 2.

The efficiency of N utilisation for milk production (milk N:N intake; NUE) varied from moderate (on average 0.25) to high (on average 0.30) in experiments I and 2, respectively. NUE generally averages around 0.25, and shows great variation between and within experiments (0.16-0.36) (Powell et al., 2010). The differences in NUE between experiments and treatments is mainly explained by the N intake (Powell et al., 2010) and sufficiency of N supply as indicated by MUN and ruminal NH_3-N concentrations. Previously, zero rumen N balance (omasal CP flow = CP intake) have been associated with an average rumen NH_3-N and MUN concentrations of 71 mg/L (equivalent of 5.07 mmol/L) and 8.3 mg/dL, respectively, and concentrations below these values might indicate a deficiency in rumen degradable protein (Broderick et al., 2010). As judged by these values, N concentration and CP:ME ratio of the diets were likely adequate in the experiment 1 as indicated by MUN concentrations of 11.8-12.9 mg/dL. In contrast, there was probably a shortage of N for rumen microbes on NEG in experiment 2 as indicated by low rumen NH_3-N (2.52 mmol/L) and MUN (6.33 mg/dL) concentrations. In NEG diet, the CP concentration of the diet was only 125 g/kg DM whereas on protein treatments it averaged 149 g/kg DM in experiment 2. Indeed, microbial N production in the rumen was increased by protein supplementation in experiment 2 signifying improved N supply in

498 this study. Even so, in 42 % of observations rumen NH_3-N concentrations on protein supplemented
499 diets were below 5.07 mmol/L.

500 The shortage of dietary N on NEG in experiment 2 plausibly lead to mobilisation of tissue protein
501 reserves as protein supplementation decreased arterial concentrations of Nτ-methylhistidine, an
502 indicator of skeletal muscle protein breakdown (Rathmacher, 2004). The concentrations of Nτ-
503 methylhistidine were also higher in experiment 2 than in experiment 1, which likely differed in dietary
504 N sufficiency. However, mobilisation of muscle N reserves was contradictory to positive N balance
505 (i.e. body N retention) in experiment 2, but potential inaccuracies in estimation of urine and faecal
506 volume from spot sampling probably impaired the reliability of calculated N balance.

507 The substitution of rapeseed supplement by spirulina in experiment 2 decreased NUE, which
508 might be explained by compensatory feeding behaviour, or protein characteristics of microalgae, such
509 as ruminal protein degradation or amino acid composition. As the concentrate proportion in the diet
510 was decreased when rapeseed meal was substituted by spirulina, larger part of the dietary N was of
511 silage origin (+0.4 kg/d silage and -0.94 kg/d concentrate on ALG compared to RSS) with sub-optimal
512 AA composition and low RUP in relation to animal requirements.

513 Spirulina inclusion in the diet increased the concentration of branched chain VFA (BCVFA) in
514 the rumen, a response that have been reported also earlier on cattle diets containing microalgae
515 (Drewery et al., 2014, Panjaitan et al., 2015, Costa et al., 2016). There are two possible explanations
516 for the response of BCVFA. First, it can be caused by the increased intake of BCAA on microalgae
517 supplemented diets, as BCAA are the substrates for BCVFA production in the rumen (e.g. El-Shazly,
518 1952, Allison, 1978). Additionally, the higher in vitro rumen degradability of spirulina CP than that
519 of rapeseed meal (Costa et al., 2016) may have further promoted the availability of BCAA for rumen
520 microbes. However, the results on the effect of rumen degradation on the formation of BCVFA are
521 inconsistent and cannot always be differentiated from the effects of AA composition of the diet
522 (Seymour et al., 1990, Rodriguez et al., 1997, Mutsvangwa et al., 2016). The suggested higher rumen

523 protein degradability of microalgae than that of rapeseed meal is supported by the observations on
524 ruminal NH_3-N concentrations, which were highest on ALG with equal N intake compared to other
525 protein supplemented diets. Further experiments are needed to confirm this, as the protein
526 degradability of some microalgae species seems to be affected by growing and cultivating conditions
527 (Lodge-Ivey et al., 2014).
528
529 *4.4. Amino acid metabolism*
530 Changing histidine supply induced varying metabolic and production responses in the present studies.
531 Protein supplementation increased the intake, and consequently also arterial concentrations of most
532 EAA, including histidine, similarly to Korhonen et al. (2002). As expected, the substitution of
533 rapeseed meal with microalgae resulted in decreases in intake and arterial concentrations of histidine.
534 Because histidine is typically the first AA limiting milk production on grass silage and cereal based
535 dairy cow diets (Kim et al., 1999, Vanhatalo et al., 1999) when milk yield exceeds 15 kg/d (Huhtanen,
536 1998), changes in histidine supply were expected to result in changes in milk production. However,
537 protein supplementation or protein source did not affect milk yields in either of the experiments which
538 contradicts the results of meta-analysis of Patton et al. (2015).
539 The unchanged milk yields, and the overall high arterial histidine concentrations compared to
540 Patton et al. (2015) or diets designed to be adequate in MP and histidine (Lee et al., 2012, Giallongo
541 et al., 2015) support the interpretation that histidine unlikely limited milk production in current
542 experiments. However, the arterial concentrations of other EAA, too, were high in current
543 experiments. The utilisation of histidine can be limited by glucose supply (Huhtanen et al., 2002), but
544 this was unlikely the case due to the relatively high arterial glucose concentrations as compared to
545 previous experiments with similar basal diets (Vanhatalo et al., 1999, Huhtanen et al., 2002).
546 On the other hand, milk protein yield and mammary histidine uptake tended to decrease when
547 microalgae substituted rapeseed meal in experiment 2, but not in experiment 1. Microalgae inclusion

548 in the diet also induced the use of endogenic histidine reserves, namely carnosine (β-alanyl-L-
549 histidine) from skeletal muscle, which is one of the metabolic adaptations to cope with the shortage
550 of histidine (Lapierre et al., 2012). Arterial carnosine concentrations decreased in both experiments
551 when microalgae substituted rapeseed supplement in the diet. Altogether, this suggests that histidine
552 supply may become suboptimal on diets containing microalgae. It is also possible that milk protein
553 yield on microalgae containing diets was limited by the overall imbalanced AA profile rather than
554 the concentrations of individual AA per se.
555
556 **5. Conclusions**
557 The suitability of non-defatted microalgae high in CP for the nutrition of lactating dairy cows was
558 demonstrated in the current experiments differing in milk production level. Although microalgae
559 inclusion in the diet did not affect DMI or milk yield in these experiments, the quality of feed intake
560 changed as cows compensated the poorer palatability of microalgae containing concentrates by
561 increasing the intake of silage. The shortage of N likely limited the growth of rumen microbes on
562 unsupplemented diet in experiment 2 as indicated by low rumen ammonia and MUN concentration.
563 Consequently, relatively high N utilisation efficiencies were observed in this experiment. With
564 similar intake of N, spirulina tended to result in higher ruminal NH_3-N concentrations than rapeseed
565 meal. This might reflect the higher ruminal protein degradability of spirulina than that of rapeseed
566 meal, the increased proportion of silage in the diets containing microalgae, or both. Despite of the
567 relatively high arterial concentrations of histidine, microalgae inclusion in the diet decreased the
568 arterial concentrations of histidine and carnosine, an endogenic histidine reserve, in both experiments,
569 and milk protein yield in experiment 2. This suggests that histidine supply of dairy cows may become
570 compromised on microalgae supplemented diets. The protein value of microalgae is likely lower than
571 that of rapeseed supplement on grass silage and cereal based dairy cow rations, as indicated by lower
572 milk protein yield, nitrogen use efficiency and calculated milk production response to increased CP

573 intake. Currently, the lack of knowledge on the feeding value of microalgae (e.g. protein degradability
574 and ruminal passage kinetics) warrants further research on utilisation of microalgae in ruminant
575 nutrition.
576
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585
586 **Appendix A. Supplementary data**
587 Supplementary data associated with this article can be found, in the online version, at
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589
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745 **Tables and figures**

746

747 **Table 1.**

748 Chemical composition (g/kg dry matter unless otherwise stated) of experimental feeds in experiments

749 1 and 2.

	Silage[1]	Cereal-sugar beet pulp[2]	Molassed sugar beet pulp	Molasses	Mineral-vitamin supplement[3]	Rapeseed supplement[4]	*Spirulina platensis*	*Chlorella vulgaris*
Experiment 1								
Dry matter, g/kg	226	891			995	871	933	946
Ash	71.1	33.8			925	70.5	68.0	57.4
Crude protein	137	130				320	687	608
Crude fat		55.2				53.0	58.7	95.1
NDF [5]	589	334				294	0	0
Starch							61.1	42.9
Experiment 2								
Dry matter, g/kg	288	899	878	710	992	866	946	
Ash	81.7	31.6	67.8	103	918	66.1	71.7	
Crude protein	133	119	113	106		311	697	
Crude fat		48.4	2.93			41.5	51.3	
NDF [5]	480	363	338			272	0	
Starch							66.2	

750 [1] Exp. 1: 66.4 g/kg dry matter (DM) of lactic acid, 11.5 g/kg DM of acetic acid, propionic and butyric
751 acid not detected, 89.2 g/kg DM of water-soluble carbohydrates (WSC), 26.7 g/kg N of NH_3-N
752 (corrected for the N added in silage preservative; uncorrected 113 g/kg N of NH_3-N), 146 g/kg DM
753 of indigestible neutral detergent fibre (iNDF), 664 g/kg DM of in vitro digestible organic matter in
754 DM (DOMD), pH 4.74. Exp. 2: 26.3 g/kg DM of lactic acid, 5.96 g/kg DM of acetic acid, 0.06 g/kg
755 DM of propionic acid, 0.21 g/kg DM of butyric acid, 147 g/kg DM of WSC, 66.9 g/kg N of NH_3-N,
756 100 g/kg DM of iNDF, 662 g/kg DM of in vitro DOMD, pH 4.28.

757 [2] Exp. 1: Contained 303 g/kg of barley, 280 g/kg of oat, 277 g/kg of barley feed, 100 g/kg of molassed
758 sugar beet pulp and 40 g/kg of molasses. Exp. 2: Contained 360 g/kg of barley, 310 g/kg of barley
759 feed, 200 g/kg of oat, 90 g/kg of molassed sugar beet pulp and 40 g/kg of molasses.

760 [3] Exp. 1 and 2: Contained 207 g/kg of Ca, 105 g/kg of Na, 60.0 g/kg of Mg, 1400 mg/kg of Zn, 500
761 mg/kg of vitamin E, 465 mg/kg of Mn, 405 mg/kg of Cu, 53 mg/kg of I, 20 mg/kg of Se, 250 000
762 IU/kg of vitamin A and 35 000 IU/kg of vitamin D_3.

763 [4] Exp. 1: Contained 767 g/kg of rapeseed meal, 108 g/kg of molassed sugar beet pulp, 75 g/kg of
764 turnip rape cake and 50 g/kg of molasses. Exp. 2: Contained 695 g/kg of rapeseed meal, 138 g/kg of
765 turnip rape cake, 117 g/kg of molassed sugar beet pulp and 50 g/kg of molasses.
766 [5] Results of silage analysed without heat stable amylase and expressed inclusive of residual ash
767 (NDF), results of concentrate components analysed with heat stable amylase and expressed inclusive
768 of residual ash (aNDF).

Table 2.

Ingredient profiles and nitrogen content of concentrates in experiments 1 and 2.

	Treatments in Experiment 1[1]			Treatments in Experiment 2[2]			
	RSS	RSS-ALG	ALG	NEG	RSS	RSS-ALG	ALG
Ingredients, kg dry mater /d							
Cereal-sugar beet pulp	7.75	8.33	8.91	10.5	7.87	8.52	9.17
Molassed sugar beet pulp						0.09	0.18
Molasses						0.03	0.06
Rapeseed supplement	2.00	1.00			2.55	1.28	
Spirulina platensis		0.23	0.47			0.57	1.13
Chlorella vulgaris		0.24	0.47				
Mineral-vitamin supplement	0.25	0.25	0.25	0.30	0.30	0.30	0.30
Total	10.0	10.1	10.1	10.8	10.7	10.8	10.9
N in concentrates, g/d	264	273	283	201	277	291	305
N in supplementary protein feed, g/d [3]	97	97	97	0	120	123	127

[1] RSS = rapeseed supplement as a protein feed; ALG = mixture of *Spirulina platensis* and *Chlorella vulgaris* (1:1 on dry matter basis) as a protein feed; RSS-ALG = mixture of RSS and ALG (1:1 on crude protein basis) as a protein feed.

[2] NEG = no protein feed; RSS = rapeseed supplement as a protein feed; ALG = *Spirulina platensis* as a protein feed, RSS-ALG = mixture of RSS and ALG (1:1 on crude protein basis) as a protein feed.

[3] The protein of rapeseed meal and turnip rape cake in rapeseed supplement was isonitrogenously substituted in half or totally by microalgae protein.

778 **Table 3.**

779 Amino acid (AA) composition (g/kg crude protein) of experimental feeds in experiments 1 and 2.

	Silage		Cereal-sugar beet pulp		Molassed sugar beet pulp	Molasses	Rapeseed supplement		*Spirulina platensis*		*Chlorella vulgaris*
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 2	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1
Essential AA											
Arginine	35.8	39.5	50.5	57.8	38.9	3.37	57.8	65.9	68.7	72.3	53.9
Histidine	11.0	17.8	19.2	23.1	27.2	2.94	25.8	28.0	16.0	15.3	17.7
Isoleucine	32.0	39.9	31.0	34.1	36.5	18.5	36.1	40.9	51.5	51.3	29.2
Leucine	58.1	73.8	58.4	66.6	56.6	17.1	63.9	74.2	81.2	82.1	68.8
Lysine	29.3	50.4	32.9	33.8	52.3	3.68	44.4	53.3	35.7	41.5	48.5
Methionine	14.8	11.6	20.5	9.6	10.3	6.30	20.4	12.9	21.4	21.9	18.8
Phenylalanine	40.3	48.1	40.6	48.7	33.5	5.55	40.8	44.7	48.1	43.9	41.8
Threonine	33.7	42.8	30.9	36.7	40.0	4.55	40.6	48.8	44.1	47.1	33.1
Tryptophan	11.3	14.5	11.4	18.3	14.3	2.19	12.4	21.8	10.9	12.4	9.66
Valine	41.3	52.5	42.9	48.5	49.2	13.4	46.8	52.6	55.6	57.0	42.6
Non-essential AA											
Alanine	56.3	65.6	39.2	43.1	48.6	23.61	38.8	46.1	65.4	70.7	60.0
Aspartic acid	61.7	84.4	60.8	64.5	62.6	30.01	61.5	76.5	72.2	88.2	55.4
Cystine	7.31	5.51	33.6	12.1	5.91	7.96	22.1	13.4	21.9	7.80	13.2
Glutamic acid	61.0	90.3	170	193	89.4	151	148	170	108	127	81.1
Glycine	38.0	48.7	38.7	46.3	40.0	15.0	48.7	55.3	48.5	47.8	46.2
Proline	38.5	49.0	64.1	78.2	41.5	12.4	56.0	62.8	34.6	35.2	37.6
Serine	32.0	42.1	38.8	46.0	43.5	8.08	40.3	48.4	45.3	46.6	31.6
Tyrosine	22.6	28.3	27.1	30.9	41.0	14.3	30.9	33.2	48.8	42.2	33.8
Σ Branched AA [1]	131	166	132	149	142	49	147	168	188	190	141
Σ Essential AA	308	391	338	377	359	77	389	443	433	445	364
Σ Non-essential AA[2]	317	414	473	514	372	262	446	506	445	466	359
Σ Total AA[3]	625	805	811	891	731	339	835	949	878	910	723

780 [1] Includes Ile, Leu and Val.

781 [2] Includes non-essential AA listed in the table.

782 [3] $\sum$ essential AA + $\sum$ non-essential AA.

783 **Table 4.**

784 Effect of substitution of rapeseed supplement by microalgae on nutrient and metabolisable energy
785 (ME) intake, nutrient digestibility, milk yield and milk composition in lactating cows in Experiment
786 1.

	Treatment[1]				Significance[2]	
	RSS	RSS-ALG	ALG	SEM	LIN	QUAD
Intake						
Silage dry matter, kg/d	12.2	12.3	12.3	0.51	0.68	0.64
Concentrate dry matter, kg/d	9.78	10.05	9.99			
Diet dry matter, kg/d	21.9	22.4	22.3	0.50	0.28	0.26
Organic matter, kg/d	20.6	21.1	21.0	0.47	0.22	0.26
Neutral detergent fibre, kg/d [3]	10.4	10.5	10.3	0.30	0.62	0.39
Crude protein, kg/d	3.30	3.43	3.46	0.070	0.009	0.23
ME intake, MJ/d [4]	233	237	236	4.7	0.18	0.24
ME balance, MJ/d [4]	30.4	33.1	37.4	9.07	0.11	0.83
Concentrate proportion	0.444	0.447	0.446	0.0109	0.80	0.81
Amino acid intake, g/d						
Arginine	147	154	156	2.6	0.001	0.17
Histidine	53.7	53.1	50.9	0.81	0.003	0.16
Isoleucine	107	113	115	2.3	0.001	0.22
Leucine	196	207	212	4.1	<0.001	0.22
Lysine	110	113	113	2.07	0.047	0.19
Methionine	58.1	60.4	61.0	1.05	0.004	0.18
Phenylalanine	134	140	143	2.8	0.002	0.22
Threonine	113	116	116	2.4	0.040	0.22
Tryptophan	38.0	38.8	38.5	0.79	0.38	0.22
Valine	141	147	149	2.9	0.003	0.21
Σ Branched AA [5]	445	467	476	9.22	0.001	0.209
Σ Essential AA	1098	1142	1154	21.7	0.004	0.20
Σ Non-essential AA[6]	1282	1323	1327	22.6	0.019	0.18
Σ Total AA[7]	2380	2465	2481	44.2	0.009	0.19
Total tract apparent digestibility, g/kg						
Dry matter	671	656	664	6.9	0.35	0.11
Organic matter	680	665	674	7.0	0.39	0.094
Neutral detergent fibre [3]	566	537	548	14.4	0.28	0.18
Crude protein	643	632	640	9.3	0.67	0.16
Yield						
Milk, kg/d	22.7	24.3	22.7	1.50	0.99	0.084
Energy corrected milk, kg/d	25.5	26.3	24.9	1.39	0.45	0.15
Fat, g/d	1120	1119	1081	70.7	0.16	0.41
Protein, g/d	848	904	831	35.0	0.71	0.14
Lactose, g/d	982	1047	985	69.7	0.96	0.15
Milk composition						
Fat, g/kg	50.4	46.2	49.8	2.75	0.84	0.12
Protein, g/kg	38.2	37.5	38.0	1.86	0.70	0.32
Lactose, g/kg	43.2	43.2	43.5	0.73	0.58	0.80
Urea N, mg/dL	12.2	11.8	12.9	0.668	0.20	0.15
Energy corrected milk (kg/d):dry matter intake (kg/d)	1.17	1.18	1.12	0.074	0.19	0.33
Milk N:N intake	0.253	0.259	0.237	0.0119	0.19	0.20

787 SEM standard error of the mean, AA amino acid.

[1] RSS = rapeseed supplement as a protein feed; ALG = mixture of *Spirulina platensis* and *Chlorella vulgaris* (1:1 on dry matter basis) as a protein feed; RSS-ALG = mixture of RSS and ALG (1:1 on crude protein basis) as a protein feed.

[2] Significance of linear (LIN) and quadratic (QUAD) components of response to substitution of rapeseed protein with microalgae protein on a grass silage based diet.

[3] Results of silage analysed without heat stable amylase and expressed inclusive of residual ash (NDF), results of concentrate components analysed with heat stable amylase and expressed inclusive of residual ash (aNDF).

[4] Silage on average 10.6 MJ/kg DM, cereal-sugar beet pulp 12.5 MJ/kg DM, rapeseed supplement 11.7 MJ/kg DM, *Spirulina platensis* 10.9 MJ/kg DM, and *Chlorella vulgaris* 11.4 MJ/kg DM.

[5] Includes Ile, Leu and Val.

[6] Includes Ala, Asp, Cys, Glu, Gly, Pro, Ser and Tyr.

[7] $\sum$ essential AA + $\sum$ non-essential AA.

Table 5.

Effect of substitution of rapeseed supplement by microalgae on arterial concentrations of plasma metabolites, amino acids (AA) and carnosine in lactating cows in Experiment 1.

	Treatment[1]				Significance[2]	
	RSS	RSS-ALG	ALG	SEM	LIN	QUAD
Plasma metabolites						
Acetic acid, mmol/L	1.46	1.40	1.58	0.101	0.41	0.34
BHBA, mmol/L	0.893	0.739	0.865	0.0412	0.55	0.011
Glucose, mmol/L	3.83	3.90	3.81	0.052	0.70	0.13
Insulin, µIU/ml	13.5	14.7	12.8	1.97	0.50	0.13
NEFA, mmol/L	0.139	0.141	0.137	0.0099	0.78	0.74
Essential AA, µmol/L						
Arginine	97.7	91.8	92.2	3.51	0.17	0.33
Histidine	56.5	56.2	50.5	6.40	0.012	0.11
Isoleucine	146	149	152	6.0	0.42	0.97
Leucine	152	152	156	6.5	0.66	0.88
Lysine	105	101	104	3.7	0.89	0.57
Methionine	26.3	26.1	24.8	1.44	0.47	0.73
Phenylalanine	53.4	53.3	52.2	1.91	0.63	0.80
Threonine	121	127	119	6.4	0.80	0.22
Tryptophan	43.9	43.5	41.6	1.02	0.16	0.57
Valine	279	278	279	12.1	1.0	0.94
Non-essential AA, µmol/L						
Alanine	225	240	235	13.5	0.36	0.32
β-alanine	23.5	23.3	23.5	2.12	1.0	0.96
Asparagine	53.2	54.3	52.9	2.50	0.94	0.67
Aspartic acid	10.5	11.7	11.5	1.35	0.61	0.71
Citrulline	74.5	73.1	71.6	2.95	0.340	1.0
Cystine	22.5	22.4	22.0	1.05	0.54	0.77
Glutamic acid	125	114	118	4.5	0.091	0.038
Glutamine	186	205	188	8.0	0.80	0.032
Glycine	292	301	272	15.6	0.014	0.008
Nτ-Methylhistidine [3]	3.84	3.88	4.29	0.476	0.20	0.51
Nπ-Methylhistidine [3]	3.85	3.56	3.68	0.274	0.56	0.41
Ornithine	52.4	51.8	51.4	2.01	0.67	0.94
Proline	84.3	86.5	85.2	4.49	0.84	0.67
Serine	94.8	93.3	89.9	4.56	0.39	0.84
Taurine	29.4	27.9	29.4	2.66	1.0	0.54
Tyrosine	49.2	49.3	49.2	2.24	1.0	0.97
Σ Branched AA[4]	577	580	587	23.6	0.73	0.93
Σ Essential AA	1080	1079	1071	34.9	0.86	0.94
Σ Non-essential AA[5]	1142	1178	1124	31.6	0.58	0.16
Σ Total AA[6]	2222	2257	2196	62.0	0.72	0.47
Carnosine	30.7	28.2	26.5	1.50	0.022	0.74

SEM standard error of the mean, BHBA β-hydroxybutyric acid, NEFA non-esterified fatty acids.

[1] RSS = rapeseed supplement as a protein feed; ALG = mixture of *Spirulina platensis* and *Chlorella vulgaris* (1:1 on dry matter basis) as a protein feed; RSS-ALG = mixture of RSS and ALG (1:1 on crude protein basis) as a protein feed.

808 [2] Significance of linear (LIN) and quadratic (QUAD) components of response to substitution of
809 rapeseed protein with microalgae protein on a grass silage based diet.
810 [3] IUPAC nomenclature. Nτ-methylhistidine = the product of muscle actin and myosin catabolism;
811 Nπ-methylhistidine = the product of anserine breakdown.
812 [4] Includes Ile, Leu and Val.
813 [5] Includes Ala, Asn, Asp, Cys, Gln, Glu, Gly, Pro, Ser and Tyr.
814 [6] $\sum$ essential AA + $\sum$ non-essential AA.

Table 6.

Effect of protein supplementation and substitution of rapeseed supplement by *Spirulina platensis* microalgae on nutrient and metabolisable energy (ME) intake, nutrient digestibility, milk yield and milk composition in lactating cows in Experiment 2.

	Treatment[1]					Significance[2]		
	NEG	RSS	RSS-ALG	ALG	SEM	PROTEIN	LIN	QUAD
Intake								
Silage dry matter, kg/d	12.2	12.9	12.8	13.3	0.57	0.071	0.43	0.54
Concentrate dry matter, kg/d	10.6	10.5	10.2	9.59				
Diet dry matter, kg/d	22.8	23.4	23.0	22.8	0.49	0.49	0.27	0.85
Organic matter, kg/d	21.2	21.6	21.3	21.2	0.45	0.64	0.29	0.86
Neutral detergent fibre, kg/d [3]	9.61	9.66	9.44	9.36	0.244	0.52	0.19	0.71
Crude protein, kg/d [4]	8.17 (2.86)	11.7 (3.42)	11.8 (3.43)	12.0 (3.45)	0.423	<0.001	0.55	0.98
ME intake, MJ/d [5]	238	245	245	249	5.1	0.036	0.33	0.57
ME balance, MJ/d [5]	17.9	16.6	22.5	24.6	6.63	0.22	0.021	0.49
Concentrate proportion	0.466	0.451	0.449	0.422	0.0152	0.034	0.044	0.31
Amino acid intake, g/d								
Arginine	136	174	180	190	3.1	<0.001	0.001	0.45
Histidine	57.8	74.6	70.9	69.2	1.45	<0.001	0.002	0.43
Isoleucine	107	133	139	149	3.3	<0.001	<0.001	0.43
Leucine	203	249	255	269	6.0	<0.001	0.004	0.43
Lysine	124	161	157	159	4.1	<0.001	0.70	0.42
Methionine	30.9	39.1	43.5	48.6	0.85	<0.001	<0.001	0.55
Phenylalanine	139	164	166	173	3.9	<0.001	0.031	0.42
Threonine	115	147	148	153	3.5	<0.001	0.080	0.43
Tryptophan [6]	1.66 (46.5)	1.77 (59.7)	1.75 (57.2)	1.75 (56.3)	0.009	<0.001	0.004	0.45
Valine	146	178	182	191	4.3	<0.001	0.006	0.42
Σ Branched AA [7]	456	560	575	609	13.6	<0.001	0.003	0.43
Σ Essential AA	1106	1380	1398	1459	31.6	<0.001	0.020	0.43
Σ Non-essential AA[8]	1315	1598	1609	1666	33.4	<0.001	0.051	0.43
Σ Total AA[9]	2421	2978	3007	3125	65.1	<0.001	0.033	0.43
Total tract apparent digestibility, g/kg								
Dry matter	637	651	646	651	5.3	0.006	0.98	0.27
Organic matter	646	660	657	661	5.5	0.003	0.76	0.38
Neutral detergent fibre [3]	441	481	475	494	11.3	<0.001	0.18	0.16
Crude protein	572	609	608	623	8.3	<0.001	0.21	0.44
Yield								
Milk, kg/d	26.7	28.0	27.3	27.3	1.02	0.22	0.38	0.60
Energy corrected milk, kg/d	28.7	30.3	29.1	29.6	1.39	0.11	0.32	0.19
Fat, g/d	1230	1299	1258	1288	75.1	0.11	0.77	0.28
Protein, g/d	999	1045	997	1002	31.5	0.36	0.059	0.17
Lactose, g/d	1109	1178	1123	1132	56.2	0.22	0.20	0.31
Milk composition								
Fat, g/kg	46.2	46.5	45.8	46.9	1.83	0.87	0.75	0.44
Protein, g/kg	37.5	37.5	36.7	36.9	0.83	0.38	0.41	0.44
Lactose, g/kg	41.4	41.9	40.7	41.2	0.74	0.82	0.21	0.095
Urea N, mg/dL	6.33	9.44	10.3	9.43	0.460	<0.001	0.99	0.028
Energy corrected milk (kg/d):dry matter intake (kg/d)	1.26	1.29	1.25	1.29	0.052	0.24	0.82	0.049

SEM standard error of the mean, AA amino acid.

[1] NEG = no protein feed; RSS = rapeseed supplement as a protein feed; ALG = *Spirulina platensis* as a protein feed; RSS-ALG = mixture of RSS and ALG (1:1 on crude protein basis) as a protein feed.

[2] Significance of protein supplementation (PROTEIN) and linear (LIN) and quadratic (QUAD) responses to substitution of rapeseed with spirulina algae on a grass silage based diet.

[3] Results of silage analysed without heat stable amylase and expressed inclusive of residual ash (NDF), results of concentrate components analysed with heat stable amylase and expressed inclusive of residual ash (aNDF).

[4] Squared transformation of crude protein intake, original values are presented in parenthesis below the squared values.

[5] Silage on average 10.6 MJ/kg DM, cereal-sugar beet pulp 12.5 MJ/kg DM, molassed sugar beet pulp 12.0 MJ/kg DM, molasses 12.6 MJ/kg DM, rapeseed supplement 11.7 MJ/kg DM, and *Spirulina platensis* 10.8 MJ/kg DM.

[6] Logarithmic transformation of tryptophan intake, original values are presented in parenthesis below the logarithmic values.

[7] Includes Ile, Leu and Val.

[8] Includes Ala, Asp, Cys, Glu, Gly, Pro, Ser and Tyr.

[9] $\sum$ essential AA + $\sum$ non-essential AA.

Table 7.

Effect of protein supplementation and substitution of rapeseed supplement by *Spirulina platensis* microalgae on rumen fermentation characteristics in Experiment 2.

	Treatment[1]					Significance[2]			
	NEG	RSS	RSS-ALG	ALG	SEM	PROTEIN	LIN	QUAD	T×D
pH	6.23	6.20	6.19	6.19	0.065	0.364	0.79	0.93	0.45
NH_3-N, mmol/L	2.52	5.32	5.38	6.37	0.661	0.001	0.11	0.37	0.040
VFA total, mmol/L[3]	97.8	101	99.5	99.2	3.46	0.189	0.43	0.77	0.53
Molar proportions, mmol/mol									
Acetate	657	658	660	655	2.4	0.879	0.34	0.21	0.13
Propionate	172	171	171	172	2.6	0.675	0.87	0.69	0.34
Butyrate	140	139	138	140	2.5	0.561	0.64	0.52	0.31
Isobutyrate	7.98	8.39	8.49	9.20	0.276	0.026	0.034	0.29	0.39
Valerate	13.3	14.2	13.5	13.8	0.30	0.153	0.40	0.27	0.11
Isovalerate	3.14	3.64	3.87	4.43	0.139	0.001	0.002	0.23	0.041
Caproate	6.72	6.13	5.94	6.40	0.179	0.022	0.28	0.14	0.36
Molar ratio									
Acetate:propionate	3.85	3.87	3.90	3.84	0.064	0.699	0.73	0.40	0.31
(Acetate+butyrate) :propionate	4.67	4.68	4.72	4.66	0.084	0.772	0.82	0.50	0.37

SEM standard error of the mean.

[1] NEG = no protein feed; RSS = rapeseed supplement as a protein feed; ALG = *Spirulina platensis* as a protein feed; RSS-ALG = mixture of RSS and ALG (1:1 on crude protein basis) as a protein feed.

[2] Significance of protein supplementation (PROTEIN) and linear (LIN) and quadratic (QUAD) responses to substitution of rapeseed with spirulina algae on a grass silage based diet and interaction of sampling time and diet (T×D).

[3] Volatile fatty acids.

Table 8.

Effect of protein supplementation and substitution of rapeseed supplement by *Spirulina platensis* microalgae on nitrogen (N) metabolism in lactating dairy cows in Experiment 2.

	Treatment[1]					Significance[2]		
	NEG	RSS	RSS-ALG	ALG	SEM	PROTEIN	LIN	QUAD
N intake, g/d [3]	209 (457)	299 (546)	303 (549)	306 (552)	10.8	<0.001	0.55	0.98
Ruminal microbial N flow, g/d [4]	305	345	321	323	12.6	0.066	0.17	0.36
Excretion in milk								
Milk N, g/d	157	164	156	157	4.9	0.36	0.059	0.17
Milk N:N intake	0.343	0.300	0.283	0.284	0.0079	<0.001	0.021	0.112
Excretion in urine								
Urine, L/d [5]	19.2	22.6	22.2	25.2	1.46	0.002	0.074	0.18
Allantoin, mmol/d	393	436	406	410	14.9	0.14	0.19	0.31
Uric acid, mmol/d	47.2	56.2	55.3	53.4	4.72	0.004	0.33	0.83
Total purine derivatives, mmol/d [6]	440	492	461	463	17.2	0.070	0.18	0.37
Urinary urea N, g/d	66.3	75.2	86.8	66.9	9.68	0.39	0.56	0.21
Urinary N, g/d	94.4	153	155	151	6.0	<0.001	0.82	0.69
Urinary urea N:urinary N	0.376	0.551	0.576	0.559	0.0134	<0.001	0.60	0.12
Urinary N:N intake	0.207	0.280	0.282	0.274	0.0105	<0.001	0.67	0.66
Excretion in faeces								
Faecal N, g/d [7]	196	214	215	208	6.20	0.013	0.47	0.49
Faecal N:N intake	0.429	0.391	0.392	0.377	0.0084	<0.001	0.21	0.44
N balance, g/d [8]	10.2	16.0	22.6	35.9	7.34	0.11	0.075	0.72

SEM standard error of the mean.

[1] NEG = no protein feed; RSS = rapeseed supplement as a protein feed; ALG = *Spirulina platensis* as a protein feed; RSS-ALG = mixture of RSS and ALG (1:1 on crude protein basis) as a protein feed.

[2] Significance of protein supplementation (PROTEIN) and linear (LIN) and quadratic (QUAD) responses to substitution of rapeseed with spirulina algae on a grass silage based diet.

[3] Squared transformation of nitrogen intake divided by 1000, original values are presented in parenthesis below the squared values.

[4] Estimated based on urinary purine derivative excretion (Puhakka et al., 2016).

[5] Estimated from urinary excretion of creatinine (Puhakka et al., 2016).

[6] Allantoin and uric acid

[7] Calculated as [1 – (apparent digestibility of N (g/kg)/1000)] × N intake (g/d)

[8] Calculated as N intake (g/d) – [N in milk (g/d) + N in faeces (g/d) + N in urine (g/d)].

862 **Table 9.**

863 Effect of protein supplementation and substitution of rapeseed supplement by *Spirulina platensis*
864 microalgae on arterial concentrations of plasma metabolites, amino acids (AA) and carnosine in
865 lactating cows in Experiment 2.

	Treatment[1]					Significance[2]		
	NEG	RSS	RSS-ALG	ALG	SEM	PROTEIN	LIN	QUAD
Plasma metabolites								
Acetic acid, mmol/L	1.60	1.44	1.56	1.55	0.100	0.33	0.31	0.57
BHBA, mmol/L	0.823	0.788	0.854	0.872	0.0496	0.72	0.096	0.57
Glucose, mmol/L	3.64	3.58	3.59	3.56	0.084	0.26	0.77	0.76
Insulin, µIU/ml	13.1	14.5	16.9	12.9	2.19	0.31	0.42	0.071
NEFA, mmol/L[3]	-1.02 (0.097)	-1.05 (0.089)	-1.01 (0.100)	-0.980 (0.114)	0.0387	0.80	0.033	0.80
Essential AA, µmol/L								
Arginine	81.3	89.2	89.7	92.9	3.32	0.012	0.38	0.70
Histidine	54.8	65.1	64.1	58.8	3.04	0.012	0.081	0.46
Isoleucine	136	149	144	150	4.55	0.030	0.87	0.32
Leucine	133	162	156	152	6.1	0.001	0.13	0.81
Lysine	106	115	115	117	3.7	0.024	0.57	0.89
Methionine	23.0	24.9	24.1	24.7	0.82	0.11	0.90	0.51
Phenylalanine	51.3	55. 1	54.3	54.2	2.05	0.096	0.70	0.88
Threonine	103	117	111	118	3.5	0.001	0.77	0.067
Tryptophan	40.1	41.5	40.3	39.4	1.22	0.86	0.23	0.91
Valine	264	309	292	292	11.6	0.003	0.18	0.42
Non-essential AA, µmol/L								
Alanine	279	266	275	276	12.3	0.18	0.48	0.75
β-alanine	4.37	3.99	3.83	4.13	0.125	0.018	0.45	0.16
Asparagine	54.7	58.6	56.7	58.7	2.21	0.19	0.99	0.45
Aspartic acid	7.82	7.62	7.86	8.26	0.487	0.87	0.37	0.90
Citrulline	64.4	68.4	66.3	69.9	2.68	0.12	0.62	0.26
Cystine	21.5	24.3	21.6	23.2	1.07	0.15	0.40	0.064
Glutamic acid	97. 7	94.7	91.6	98.4	4.16	0.53	0.50	0.31
Glutamine	220	221	210	223	7.65	0.76	0.80	0.13
Glycine	360	327	329	337	20.2	0.062	0.60	0.83
Nτ-Methylhistidine[4]	6.40	5.37	5.03	5.10	0.333	<0.001	0.29	0.35
Nπ-Methylhistidine[4]	4.39	3.47	3.31	3.53	0.333	<0.001	0.82	0.37
Ornithine	52.7	57.2	57.7	57.8	2.29	0.040	0.83	0.92
Proline	89.7	95.3	88.8	87.7	3.01	0.76	0.051	0.39
Serine	91.7	98.1	93.8	96.2	4.69	0.30	0.70	0.45
Taurine	40.8	44.2	42.1	41.3	1.97	0.40	0.25	0.75
Tyrosine	45.5	51.5	51.3	51.0	2.63	0.015	0.85	1.0
Σ Branched AA[5]	532	620	591	593	20.6	0.002	0.26	0.45
Σ Essential AA	991	1127	1090	1099	28.0	0.002	0.43	0.47
Σ Non-essential AA[6]	1440	1427	1403	1441	45.3	0.66	0.76	0.45
Σ Total AA[7]	2259	2371	2316	2358	51.4	0.087	0.83	0.36
Carnosine	27.1	27.0	24.2	21.7	1.92	0.059	0.006	0.94

866 SEM standard error of the mean, BHBA β-hydroxybutyric acid, NEFA non-esterified fatty acids.

867 [1] NEG = no protein feed; RSS = rapeseed supplement as a protein feed; ALG = *Spirulina platensis*
868 as a protein feed; RSS-ALG = mixture of RSS and ALG (1:1 on crude protein basis) as a protein feed.

[2] Significance of protein supplementation (PROTEIN) and linear (LIN) and quadratic (QUAD) responses to substitution of rapeseed with spirulina algae on a grass silage based diet.

[3] Logarithmic transformation of arterial NEFA concentration, original values are presented in parenthesis below the logarithmic values.

[4] IUPAC nomenclature. Nτ-methylhistidine = the product of muscle actin and myosin catabolism; Nπ-methylhistidine = the product of anserine breakdown.

[5] Includes Ile, Leu and Val.

[6] Includes Ala, Asn, Asp, Cys, Gln, Glu, Gly, Pro, Ser and Tyr.

[7] $\sum$ essential AA + $\sum$ non-essential AA.